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EVALUATION OF BLOOD TESTING METHODOLOGY FOR USE IN SPORT

PROCJENA METODOLOGIJE TESTIRANJA KRVNOG DOPINGA U SPORTU

Harm Kuipers[1,2], Tim Brouwer[1], Steven Vanhoutvin[1], Sanda Dubravcic-Simunjak[2], Jane Moran[2], David Mitchel[2], Joel Shobe[2], Hiroya Sakai[2], and James Stray-Gundersen[2]

[1]Dept. of movement sciences, niversity of Maastricht, The Netherlands
[2] International Skating Union, Lausanne, Switzerland

ABSTRACT

Aim: to study the effect of posture, duration of storage at room temperature (20°C) or in the refrigerator (8°C), time of the day, as well as intra-sample variability and possible differences between two hematological analyzers, on hematological variables.

Methods: in 13 healthy subjects we conducted a series of experiments**.** For inter-analyzer comparison a number of samples was analyzed both on an Advia-120 and on a Gen-S analyzer.

Results: comparing standing and 5 minutes sitting, no difference in hemoglobin (Hb) and hematocrit (Ht) was found. However, the number of reticulocytes was lower ($p<0.01$) after 5 minutes sitting ($44.1\pm6.5 \times 10^9$/l) compared to standing ($69.1\pm4.8 \times 10^9$/l). Storage at room 20°C or 8°C for 8 hours did not affect hemoglobin or hematocrit, although the mean number of reticulocytes increased from $58.3\pm22.3 \times 10^9$/l to $74.2\pm21.0 \times 10^9$/l after 8 hours in both cases. Hematological variables did not change significantly during the day, except for the number of reticulocytes that peaked at 5 pm ($78.1\pm7.3 \times 10^9$/l) compared to 8 am ($57.5\pm7.1 \times 10^9$/l). On the Advia 120 (Bayer) hemoglobin, hematocrit, and number of reticulocytes were slightly but consistently higher ($p<0.001$) compared to the General-S (Coulter).

Conclusion: before blood draw in sport, the athletes should be seated for 5 minutes. For reticulocyte count the samples should preferably be analyzed within 8 hours at unique analizator.

Key words: sport, blood testing, hematological variables

SAŽETAK

Cilj: istražiti utjecaj posture, trajanja spremljenosti uzoraka na sobnoj temperaturi (20°C) ili u hladnjaku (8°C), vremena uzimanja uzoraka tijekom dana, varijabilnost među uzorcima te moguće razlike između 2 hematološka analizatora na testirane hematološke varijable.

Metode: na 13 zdravih ispitanika izvedena je serija ispitivanja. Za ispitivanje razlika između 2 hematološka analizatora dio uzoraka analiziran je na Advia 120 i na Gen-S analizatoru.

Rezultati: uspoređujući vrijednosti hematoloških varijabla nakon uzimanja uzorka ispitanika kada je stajao i kada je 5 minuta sjedio, nisu nađene razlike u vrijednostima hemoglobina i hematokrita. Međutim, broj retikulocita bio je niži ($p<0.01$) nakon što je ispitanik 5 minuta sjedio ($44.1\pm6.5 \times 10^9$/l) u usporedbi s ispitanim brojem retikulocita nakon stajanja ($69.1\pm4.8 \times 10^9$/l). Spremanje uzorka tijekom 8 sati na sobnoj temperaturi od 20°C ili u hladnjaku na 8°C nije utjecalo na vrijednosti hemoglobina i hematokrita, ali je broj retikulocita porastao s $58.3\pm22.3 \times 10^9$/l na $74.2\pm21.0 \times 10^9$/l u oba slučaja. Vrijednosti ispitanih hematoloških varijabli nisu se značajno mijenjale tijekom dana, osim što je broj retikulocita imao svoju maksimalnu vrijednost u 17 sati ($78.1\pm7.3 \times 10^9$/l) uspoređujući je s vrijednostima uzetih u 8 sati ujutro ($57.5\pm7.1 \times 10^9$/l). Vrijednosti hemoglobina, hematokrita i broja retikulocita bile su statistički značajno više ($p<0.001$) kada su testirane na Advia 120 analizatoru u usporedbi s njihovim vrijednostima dobivenim testiranjem na General-S (Coulter).

Zaključak: prije uzimanja uzoraka krvi u športaša za krvni doping, športaši trebaju sjediti 5 minuta, a vezano uz vrijednosti retikulocita uzorak treba testirati unutar 8 sati od njegovog uzimanja na jedinstvenom analizatoru.

Ključne riječi: sport, testiranje krvnog dopinga, hematološke varijable

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INTRODUCTION

In endurance sports a high oxygen uptake capacity is a prerequisite for athletic success. In an attempt to increase the maximal oxygen uptake via enhancement of the oxygen transport capacity of the blood, blood doping has been proven to be effective (3, 16). A more recent development is the administration of recombinant human erythropoietine (rh-EPO) which enhances the erythrocyte production. Studies have shown that an increase of the hemoglobin mass significantly increases maximal oxygen uptake (1, 5). Because of its potential to enhance performance the International Olympic Committee (IOC) has put rh-EPO on the list of banned substances. However, rh-EPO is difficult to detect, and can only be found in the urine within approximately 5 days after the last injection (9). Therefore it has been attempted to look also for the effects of rh-EPO, i.e. hematocrit, hemoglobin concentration and/or indirect markers of increased erythropoiesis such as number of reticulocytes and reticulocytic characteristics (10, 11, 12, 13). For this purpose, some international sport federations introduced blood tests for measurement of some key hematological variables. The International Cycling Union (UCI) introduced measurement of hematocrit in cyclists, and above a hematocrit level of 50% and 47%, male and female cyclists, respectively are not allowed to start (14). The International Ski Federation (FIS), and International Biathlon Union (IBU) use hemoglobin concentration. Above a hemoglobin concentration of 17.5 g/dl and 16.0 g/dl male and female athletes, respectively, are not allowed to start (4, 6).

The International Skating Union (ISU) started in 1999 with a blood testing program, which is an extension and refinement of the blood testing methods used so far (7). With this program 3 ml of blood is collected from all athletes one day prior to selected events, and in addition to hemoglobin and percentage reticulocytes, also other parameters for erythropoiesis are determined such as the size of erythrocytes (MCV in fl), cellular hemoglobin concentration (MCH in pg), and reticulocyte hemoglobin concentration (2, 11). The aim of this approach is to obtain a data set which enables targeted urine testing for EPO in case of significant changes in erythropoiesis and/or hemoglobin concentration. Similar to other international sport federations above a hemoglobin level of 18.0 g/dl in man and 16.5 g/dl in women, a skater is not allowed to start (7).

A no start has great impact on the athlete, and therefore it is important to have the methodology standardized and as refined as possible. In order to have a solid basis for a start or no-start decision, factors that need to be established are for instance reproducibility of the measurements, the effect of posture, duration of tourniquet, and time of the day.

Therefore, the general aim of the present study was to investigate the effect of some important pre-analytical factors on selected hematological variables. More specifically in the present study we investigated:

1) the effect of duration of sitting and standing on the measured parameters
2) the effect of storage of the samples either at room temperature or refrigerated on the measured hematological variables
3) the variability of hematological variables in samples taken at different times during the day
4) the variability of repeated measurements from the same sample on the selected hematological variables
5) possible differences in some key hematological variables in the same sample, analyzed on two different hematological analyzers.

MATERIALS AND METHODS

Subjects

13 students, who were all healthy, and physically active (range 20-26 years) volunteered for this study. Before participation the subjects were medically screened by interview with a physician, and they signed an informed consent.

The subjects were informed and fully aware of the purpose and the nature of the study, and also of possible risks and inconveniences associated with the study, which was a science project that the students chose to do as part of an elective program for the university master program in health sciences.

Blood collection

Before venipuncture the subjects were sitting on a chair for five minutes, except for the experiment in which the duration of sitting was studied.

For blood sampling an antecubital vein was punctured and 3 ml of blood was sampled with a vacuum system, using K3-EDTA Vacutainer tubes (Sherwood medical, UK). Before blood sampling a tourniquet with moderate pressure was applied, and 5-10 seconds elapsed before the blood started to run. As soon as the blood started to run, the tourniquet was released.

Experimental design

The subjects were subjected to different experiments on different days. Between two experiments an interval of at least 3 days was kept.

For participating in an experiment the subjects reported to the laboratory around 8 am after having consumed a normal breakfast. During the day, except for the moments of blood sampling the subjects maintained their regular daily activities.

Experiment 1

Ten subjects participated in this experiment in which the effect of sitting or standing on hematological variables was investigated. After reporting to the lab the subjects stood for 10 minutes after which a blood sample was taken. Subsequently the subjects sat down, and after 1 and 5 minutes sitting a blood sample was taken again. After inserting the needle for the first sample the needle was left in place between subsequent sampling. Before every sample the tourniquet was tightened until the blood started to run.

In four out of the 10 subjects blood was sampled more frequently (after 1, 3, 5, 7, 9, 11, 13, and 15 minutes).

Experiment 2

Ten subjects participated in this experiment, in which the effect of both time after sampling and of storage of the blood samples at room temperature or in the refrigerator was studied. After the subjects sat for 5 minutes, in every subject 6 tubes of blood were sampled. Two tubes were analyzed within 30 minutes after sampling, and 2 tubes

were put immediately in the refrigerator and kept there at 8±0.3°C, while 2 tubes were kept at room temperature (20 °C±1°C). At 4 and 8 hours after sampling one sample kept refrigerated and one stored at room temperature were analyzed.

Experiment 3

Thirteen subjects participated in this experiment in which the three goals were 1) to study the variability between samples taken at the same time, and 2) to study possible changes during the day in any of the hematological variables, and 3) to compare the same samples analyzed on two different type hematological analyzers.

In every subject 3 tubes of blood were sampled between 8 and 8.15 am, and subsequently three tubes of blood were sampled every three hours, until 5.15 pm. Before taking blood the subjects sat down for 5 minutes after which the tourniquet was applied and the three samples were taken. Every time 2 tubes taken at the same time were analyzed on the Advia-120 for studying inter-sample variability, and in 10 subjects also on the General-S (Counter, Miami, USA) for comparison of the two analyzers.

Analysis and variables

The K3-EDTA tubes (Sherwood medical, UK) were placed on a blood mixer for 5 minutes prior to analysis by the Advia 120 (Bayer Corporation, Tarrytown, NY, USA). For calibration the Sport Calibration procedure was utilized (8).

For comparison of Hb, Ht and number of reticulocytes on another hematological analyzer, as series of samples was analyzed on a Coulter Gen-S (Counter, Miami, USA).

Data handling and statistical analysis

The data were stored in an excel file, and analyzed using a statistical package (SPSS). To compare samples at various time points of the day a repeated measures ANOVA was employed. For comparison of duplicate measurements, as well as to compare the data measured on two different machines a paired t-test was used. When using analysis of variance (ANOVA) a post-hoc test was done to locate differences. A difference was considered statistically significant at $p<0.05$. Data are presented as means ± standard deviation.

RESULTS

As can be seen in Table 1, duplicate measurement in 30 samples, analyzed on the Advia-120 do not show significant difference in hemoglobin concentration, hematocrit, and number of reticulocytes. In none of the additional measured parameters significant differences were found between duplicate measurements.

When comparing the effect of standing and sitting, it was found that hemoglobin concentration (Hb) and hematocrit (Ht) were 15.5±1.0 mmol/l and 46.7±2.5 %, during standing and 15.3±0.8 mmol/l and 46.9±2.3 % after sitting for 5 minutes, respectively ($p>0.05$). In the standing position the number of reticulocytes was 69.1±4.8 x 10^9/l, and 44.1±6.5 x 10^9/l after 5 minutes sitting ($p<0.01$), respectively. Calculated reticulocyte Hb was higher during standing (2.2 g/l), compared to sitting (1.4 g/l; $p<0.01$). In 4 of the subjects blood was sampled up to 15 minutes sitting. The data show that from 5 minutes sitting the hematological variables did not show significant changes and remained stable throughout the 15 minutes sitting.

Table 1 Mean values of duplicate measurements of hemoglobin (Hb), hematocrit (Ht), number of reticulocytes (#retics) in number (x 10^9/l). Mean values standard deviation is presented. The number (1 or 2) indicates first and second measurement of the same samples.

Tablica 1 Srednje vrijednosti dvostrukih mjerenja hemoglobina (Hb), hematokrita (Ht), broja retikulocita (#retics) u brojevima (x 10^9/l). Srednje vrijednosti standardne deviacije su navedene. Brojevi (1 ili 2) označavaju prvo i drugo mjerenje istog uzorka.

Hb1 (g/dl)	15.4 ± 0.8	Hb2 (g/dl)	15.4 ± 0.9
Ht1 (l/l)	0.46 ± 2.2	Ht2 (l/l)	0.46 ± 2.2
#retics1 (x 10^9/l)	59.2 ± 21.3	#retics2 (x 10^9/l)	61.0 ± 20.7

Comparing storage of the samples at room temperature or refrigerated showed that Hb and Ht did not change during the 8 hours storage in either condition. However, the number of reticulocytes increased significantly over the 8 hours storage in both storage conditions. Storage in the refrigerator showed that the number of reticulocytes increased from 58.3±22.3 x 10^9/l immediately after sampling to 74.2±23.6 x 10^9/l. After storage at room temperature the number of reticulocytes increased to 74.6±23.6 x 10^9/l after 8 hours ($p<0.001$ between pre-storage and 8 hours storage). A remarkable observation was that in the refrigerator the increase in number of reticulocytes occurred during the first 4 hours and increased from 58.2±22.3 x 10^9/l immediately after sampling to 71.2±17.4 x 10^9/l after 4 hours storage ($p<0.05$). At room temperature the increase mainly occurred between 4 and 8 hours of storage. The calculated reticulocyte hemoglobin concentration increased from 1.9±0.7 g/l immediately after draw to 2.4±0.7 g/l after 8 hours storage at room temperature and to 2.3± 0.6 g/l after 8 hours storage at 8°C ($p<0.01$).

When taking blood samples at different time points during the day, the results show that Hb and Ht do not change significantly during the day, unlike the number of reticulocytes, and consequently reticHb. The number of reticulocytes increased from 57.4±7.1 x 10^9/l at 8 a.m. to 78.1±7.3 at 5.15 pm ($p<0.05$). Also reticulocyte hemoglobin increased from 1.8±0.2 g/l to 2.5±0.2 g/l during the day ($p<0.05$).

Comparing the hematological variables measured on the Advia 120 and on the Gen-S (Coulter) demonstrates that Ht values are significantly higher ($p<0.001$) on the Advia (45.4±2.2) compared to the Gen-S (43.5±2.2). In addition hemoglobin concentration measured on the Advia-120 is significantly ($p<0.001$) higher (15.4±1.3 g/dl) than on the Gen-S (14.9±1.3 g/dl). Also the mean number of reticulocytes on the Advia 120 (78.0±18.1 x 10^9/l) is significantly higher ($p<0.001$) compared to the Gen-S (34.5±14.2 x 10^9/l).

DISCUSSION

The present study aimed to investigate pre-analytical factors on hematological variables in healthy volunteers.

It was found that hemoglobin concentration and hematocrit were not significantly different between standing and sitting for 5 minutes, suggesting that changes in posture do not affect blood composition under these conditions. Also in the four subjects who were measured until 15 minutes sitting, no changes in hematological variables were observed throughout the 15 minutes sitting. A remarkable finding was that in contrast to hemoglobin and hematocrit, the number of reticulocytes did change, i.e. decreased after 5 minutes in the sitting position. The remarkable, quick posture-dependent decline in number of reticulocytes is difficult to explain, and no similar studies have been conducted which might enable proper comparison.

An important point for blood testing in sport is whether hematological variables in the sample may change over time, because in sport practice the samples may have to be transported from the site of blood sampling to the laboratory, and analysis can take place only a few hours after sampling. The present study shows that up to 8 hours after sampling storage at room temperature or at keeping the samples refrigerated at 8 $^{\circ}$C does neither affect hemoglobin concentration nor hematocrit. However, when stored at room temperature the number of reticulocytes increased significantly between 4 and 8 hours storage. When the samples were kept refrigerated the number of reticulocytes increased significantly after 4 hours storage, after which no further change was observed. Since no comparable studies have been published, the finding cannot be compared. It is speculated that the change in reticulocyte number during storage may be caused by changes in staining properties over time. However this finding requires further investigation. Also the finding that the change in number of reticulocytes when stored at room temperature changes after 4 hours, whereas storage in the fridge changes the number of reticulocytes during the first 4 hours, is unexplained. The change in reticulocyte number during the two storage conditions suggests that any effect on staining properties is temperature dependent. The results of this study appear to contrast those of Parisotto et al (14), who stated that the hematological variables would remain stable until 4 days after sampling when kept at 4°C. However, the statement of Parisotto is not substantiated with data. It cannot be ruled out that storage at 8 ^{0}C may yield different results than storage at 4 ^{0}C. Based on the results of the present study it appears that possible changes in hematological variables should be taken into account when interpreting results from samples that have been stored for some hours.

For blood testing in sport it is relevant to know in how far hematological variables may change during the day. The present study shows that hematocrit and hemoglobin concentration do not change during the day, unlike the number of reticulocytes, which increased significantly between 3 and 5 pm, compared with previous samples. Since no comparable studies are available this finding deserves further study. The absence of changes in hemoglobin concentration and hematocrit during the day are in line with the study by Schmidt et al (13), who also found stable hemoglobin concentrations and minor changes in hematocrit during the day. However, Schmidt et al (13) did observe a significant decline during the night in both hemoglobin concentration and hematocrit. Because in the present study no measurements were done during the night this finding cannot be substantiated. Unfortunately no other studies are available about possible changes in number of reticulocytes during the day .

Blood testing in sport is not always done on the same type of hematological analyzer, and therefore it may be questioned in how far results from one machine can be compared with those obtained on another machine. The data from the present study show that analysis of the blood same sample on two different hematological machines may yield different results. On the Advia 120 (Bayer) hemoglobin concentration and hematocrit are significantly higher than on the Gen-S (15.4±1.3 g/dl on the Advia 120 versus 14.9±1.3 g/dl on the Gen-S). Since no cross calibration was done, the differences may be attributable to different calibration procedures. This is also true for the number of reticulocytes, which are significantly and consistently higher on the Advia 120 (78.0±18.1 x 10^{9}/l) compared to the Gen-S (34.5±14.2 x 10^{9}/l). This difference is larger than reported in the study by Vandenbossche et al (15). The difference in reticulocyte count may be attributable to differences in analysis technology of both machines. However, these possible differences have been reported but should be taken into account when interpreting and comparing data obtained on different analytical systems (15).

In summary, the study shows that the various variables do not change during the day, except for the number of reticulocytes that may change at the end of the afternoon. For standardization of blood sampling in sport 5 minutes sitting appears to be sufficient for stabilization of the hematological variables, while the application of a tourniquet should be less than one minute. Storage for up to 8 hours does not affect hemoglobin and hematocrit, however, after 4 hours storage the number of reticulocytes may increase. The study shows that analysis on different hematological machines may yield differences in the results that have to be taken into account when attempting to harmonize the blood testing procedures in sport.

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